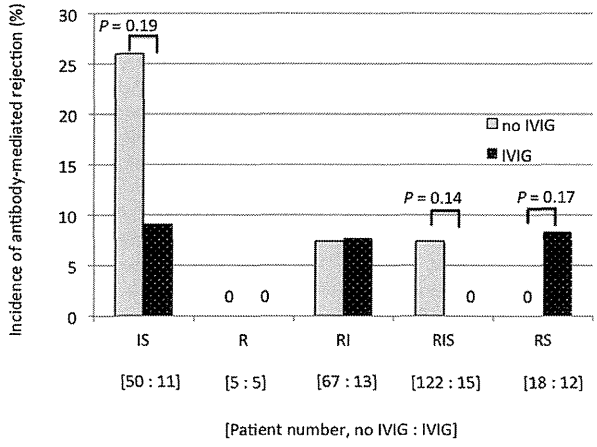

Figure 6: Comparison of the incidences of antibody-mediated rejection (AMR) with and without intravenous immunoglobulin (IVIG) in each regimen. IS, local infusion with splenectomy without rituximab; R, rituximab without splenectomy or local infusion; RI, rituximab with infusion but without splenectomy; RIS, rituximab with both infusion and splenectomy; RS, rituximab with splenectomy but without infusion. There were no significant differences in the incidence of AMR.

infections and CMV disease after transplantation were similar between the nonrituximab and rituximab groups, but the incidence of fungal infection was significantly lower in the rituximab group. Although data for the amount of steroid and trough levels of calcineurine inhibitors were not collected here, the total amount of conventional immunosuppressant might be reduced in light of the expected beneficial effects of rituximab. Lower amounts of conventional immunosuppressants might be a reason for the lower fungal infections.

In this study, half the patients were given 500 mg/body, a quarter were given 300 mg/body and a quarter were given 375 mg/m^2 (corresponding to 430–762 mg/body; median, 600 mg/body). One reason for dose reduction could be concern about potential adverse effects in patients with end-stage liver diseases. In kidney transplantation, Shirakawa et al (20) reported a successful trial to reduce rituximab from 500 to 200 mg/body. Here, there was a tendency toward a higher incidence of AMR in patients treated with $\leq$300 mg/body compared with 500 mg/body or 375 mg/m^2; however, three patients treated with 130 mg/body or 200 mg/body belonged to the same center, and one of them died from severe AMR. More evidence is needed before we can recommend reducing the rituximab dose below 300 mg/body in liver transplantation.

Multiple administrations of rituximab are standard in the treatment of B cell lymphoma. However, because the amount of targeted B cells is expected to be much smaller in transplant patients, a single dose is usually applied. A single dose is standard in kidney transplantation. Here, there were patients with two administrations in six centers and with three administrations in three centers, but the majority of these patients underwent transplantations in 2010 or earlier. All three centers changed their policy to one dose in 2012 on the basis of our data. The current study clearly demonstrates that multiple doses provide no significant benefit in terms of AMR incidence or survival, whereas they increase the incidences of fungal and CMV infections.

The Kyoto group recommended early administration of rituximab to deplete B cells, although the incidence of clinical AMR did not increase significantly in patients with late administration (2). Here, the timing of rituximab administration had no significant effect on AMR incidence on patient survival. Furthermore, 6 of 22 patients with FHF were given rituximab within 6 days before transplantation and survived without AMR. Hence, administration of rituximab immediately before transplantation is a promising therapeutic strategy.

The titers decrease after desensitization before transplantation and increase or do not change immediately after transplantation, and they usually decrease thereafter when patients survive (1). Hence, the optimum cut-off values vary among time points, between IgM and IgG. In rituximab-treated patients, peak IgG and IgM DSA titers posttransplantation were significantly greater in those with AMR, and the AMR incidence was significantly higher in patients with peak titers posttransplantation above optimum cut-off values calculated from ROC curves (i.e. IgM, $\geq$64; IgG, $\geq$128). Theoretically, it is an option to treat patients preemptively by using other desensitization methods such as IVIG and plasmapheresis when antibody titers are above the cut-off values; however, the decision is still difficult.

This study had limitations. It was an uncontrolled retrospective observational study with many confounders, some of which may have been nonrandom and unaccounted for, and thus despite the use of appropriate multivariate statistics unknown bias was possible. Because of the extent of co-linearity between rituximab and era, estimates of regression coefficients still might be unstable, although we tried to adjust era effects as much as possible. Prospective studies are required to examine the causality of the relationships found.

In conclusion, outcomes in adult ABO-I LDLT have significantly improved in the latest era coincident with the introduction of rituximab.

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Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

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Case Report

Two patients treated with pegylated interferon/ribavirin/telaprevir triple therapy for recurrent hepatitis C after living donor liver transplantation

Tomokazu Kawaoka,[1] Shoichi Takahashi,[2] Yumiko Tatsukawa,[1] Akira Hiramatsu,[1] Nobuhiko Hiraga,[1] Daiki Miki,[1] Masataka Tsuge,[1] Michio Imamura,[1] Yoshiiku Kawakami,[1] Hiroshi Aikata,[1] Hidenori Ochi,[1] Kouhei Ishiyama,[3] Kentaro Ide,[3] Hirotaka Tashiro,[3] Hideki Ohdan[3] and Kazuaki Chayama[1]

[1]*Department of Gastroenterology and Metabolism, Hiroshima University Hospital,* [2]*Department of Gastroenterology, Kouyou Nyutaun Hospital, and* [3]*Department of Surgery, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Science, Hiroshima University, Hiroshima, Japan*

It is difficult to use protease inhibitors in patients with recurrent hepatitis C virus (HCV) infection after liver transplantation (LT) due to interaction with immunosuppressive drugs. We report our experience with two patients treated with telaprevir (TVR) combined with pegylated interferon/ribavirin (PEG IFN/RBV) for recurrent HCV genotype 1 infection after LT. The first was a 63-year-old man with HCV-related liver cirrhosis, who failed to respond to IFN-β plus RBV after LT. Treatment was switched to PEG IFN-α-2b plus RBV and TVR was started. The donor had TT genotype of interleukin (IL)-28 single nucleotide polymorphisms (SNP) (rs8099917). The recipient had TT genotype of IL-28 SNP (rs8099917). Completion of 12-week triple therapy was followed by PEG IFN-α-2b plus RBV for 36 weeks. Finally, he had sustained viral response. The second was a 70-year-old woman with HCV-related liver cirrhosis and hepatocellular carcinoma. She failed to respond to PEG IFN-α-2b plus RBV after LT, and was subsequently switched to PEG IFN-α-2b/RBV/TVR. Genotype analysis showed TG genotype of IL-28 SNP for the donor, and TT genotype of IL-28 SNP for the recipient. Serum HCV RNA titer decreased below the detection limit at 5 weeks. However, triple therapy was withdrawn at 11 weeks due to general fatigue, which resulted in HCV RNA rebound 4 weeks later. Both patients were treated with cyclosporin, starting with a small dose to avoid interactions with TVR. TVR is a potentially suitable agent for LT recipients who do not respond to PEG IFN-α-2b plus RBV after LT.

Key words: hepatitis C virus, liver transplantation, telaprevir

INTRODUCTION

THE HEPATITIS C virus (HCV) has infected 170 million people worldwide, which progresses in some patients to liver cirrhosis and/or hepatocellular carcinoma (HCC).[1] The current treatment for patients infected with HCV genotype 1 is the combination of pegylated interferon-α and ribavirin (PEG IFN/RBV) for 48 weeks.[2] However, this treatment produces sustained viral response (SVR) in only approximately 50% of patients with genotype 1 HCV infection. In 2011, the first direct-acting antiviral agent (DAA) for the treatment of HCV genotype 1, telaprevir (TVR), was approved and treatment with this agent improved SVR to approximately 70–80% of patients with genotype 1 HCV infection.[3,4]

Recurrence of HCV infection after liver transplantation (LT) is one of the major causes of morbidity and allograft loss after LT.[5,6] Because the outcome of post-LT therapy with the classic antiviral agents PEG IFN/RBV are at most moderate with respect to SVR, LT patients constitute one of the classic difficult-to-treat groups.[7–9] The newly introduced triple therapy of protease inhibitors (PEG IFN/RBV/TVR) offers promising perspectives

Correspondence: Dr Tomokazu Kawaoka, Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan. Email: kawaokatomo@hiroshima-u.ac.jp
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for the management of LT patients, although TVR is not yet approved for use in LT patients.

Although there is urgent need for effective treatment of HCV recurrence after LT, significant concern has been expressed about the safety and efficacy of HCV protease inhibitors in this setting because of the side-effect profile and the potential for drug–drug interactions with immunosuppressive agents.[10] Both cyclosporin and tacrolimus are substrates of cytochrome P450 3A and P-glycoprotein. Thus, co-administration of TVR, a potent cytochrome P450 3A4 substrate and inhibitor with the potential to saturate or inhibit intestinal P-glycoprotein, substantially increases the blood levels of cyclosporin and tacrolimus.[11] Consequently, the blood concentration of tacrolimus increased 78-fold, and that of cyclosporin increased fourfold by interaction with TVR.[11] In their recent pilot study, Werner *et al.*[10] described the response to 12-week treatment with TVR plus tacrolimus, cyclosporin or sirolimus in nine patients. Pungpapong *et al.*[12] also reported the preliminary data of 35 patients treated with TVR plus cyclosporin and those of another group of 25 patients treated with boceprevir. Here, we report our preliminary data on protease inhibitors used in combination with PEG IFN/RBV for the treatment of recurrent HCV genotype 1 infection after LT.

CASE REPORT

Case 1

THIS PATIENT WAS a 63-year-old man with HCV-related liver cirrhosis. Living donor LT (LDLT) was performed after obtaining informed consent at May 2009. In August 2009, the patient was started on IFN-β (600 μg) plus RBV (200 mg) due to depression. Because serum HCV RNA titer never fell below the detection limit (1.2 log IU/mL) over the 48-month treatment period, tacrolimus was switched to cyclosporin. In April 2012, treatment was changed to PEG IFN-α-2b (100 μg) plus RBV (200 mg, due to anemia) and TVR (1500 mg) because of depression. At the start of triple therapy, the platelet count was 24.6×10^4/μL, alanine aminotransferase (ALT) was 45 IU/L, genotype was 1b and HCV RNA was 6.8 log IU/mL. Further analysis showed six amino acid (a.a.) substitutions in interferon sensitivity-determining region (ISDR), and mutant- and wild-type amino acids at a.a.70 and a.a.91 in the core region, respectively. The donor had TT genotype of IL-28 single nucleotide polymorphisms (SNP) (rs8099917) and TT/TT genotype of λ4 (ss469415590). The recipient had TT genotype of interleukin (IL)-28 SNP (rs8099917) and TT/TT genotype of λ4 (ss469415590) (Table 1, Fig. 1). Cyclosporin was started at 10 mg/day after triple

Table 1 Laboratory data of patient 1 at start of triple therapy after LT

CBC		LDH	219 IU/L	Tumor marker	
WBC	4630/μL	ALP	357 IU/L	AFP	4.8 ng/mL
RBC	4.01×10^6/μL	γ-GT	20 IU/L		
Hb	12.4 g/dL	TP	7.3 g/dL	HCV virus markers	
Ht	37.8%	Alb	4.0 g/dL	HCV RNA	6.8 KIU/mL
Plt	24.6×10^4/μL	TC	164 mg/dL	Genotype	1b
		TTT	12 U		
Blood coagulation test		ZTT	15 U		
PT	120%	BUN	24.6 mg/dl	a.a. substitution in ISDR	6
		Cr	1.07 mg/dl	a.a.70 in the core region	Mutant
Blood chemistry		CRP	0.10 mg/dl	a.a.91 in the core region	Wild
T-Bil	0.5 mg/dL	NH_3	32 μg/mL	*IL-28B* donor	TT genotype
AST	30 IU/L			*IL-28B* recipient	TT genotype
ALT	45 IU/L			ss469415590 donor	TT/TT genotype
FBS	98 mg/dL			ss469415590 recipient	TT/TT genotype
HbA1c	5.5%			AUC of telaprevir	103 μgh/mL

γ-GT, γ-glutamyltransferase; a.a. substitution in ISDR, amino acid substitutions in the interferon sensitivity-determining region; AFP, α-fetoprotein; Alb, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUC, area under curve; BUN, blood urea nitrogen; CBC, complete blood count; Cr, creatinine; CRP, C-reactive protein; FBS, fasting blood sugar; Hb, hemoglobin; HbA1c, hemoglobin A1c; Ht, hematocrit; LDH, lactate dehydrogenase; LT, liver transplantation; RBC, red blood cells; Plt, platelets; PT, prothrombin time; T-Bil, total bilirubin; TC, total cholesterol; TP, total protein; TTT, thymol turbidity test; WBC, white blood cells; ZTT, zinc sulfate turbidity test.

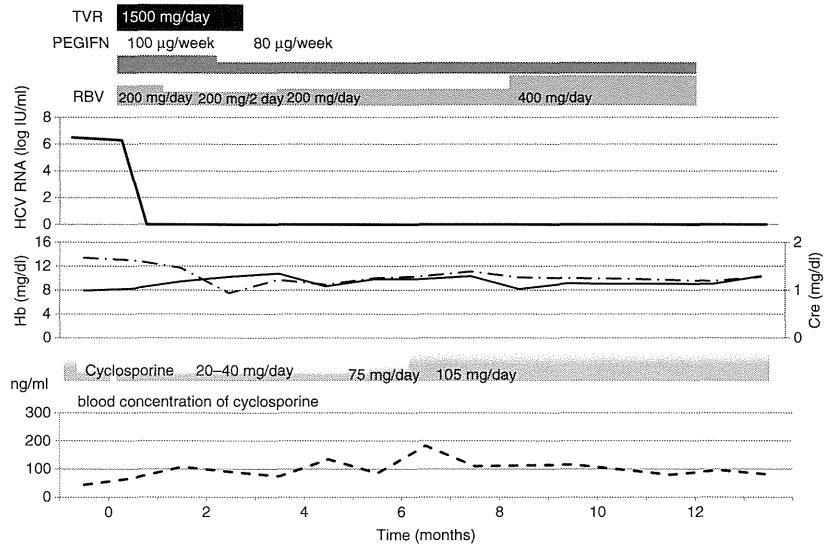

Figure 1 Clinical course of patient 1. Cre, creatinine; Hb, hemoglobin; HCV, hepatitis C virus; PEG IFN, pegylated interferon; RBV, ribavirin; TVR, telaprevir. ——, Cre; — ·, Hb.

therapy, but subsequently increased (based on measurement of its level in the peripheral blood during follow up) to 105 mg/day. The area under the curve (AUC) of TVR was 103 μgh/mL. Serum HCV RNA titer fell below the detection limit (1.2 log IU/mL) at 2 weeks after triple therapy. After 12-week triple therapy, PEG IFN-α-2b and RBV were continued for 36 weeks until April 2013. Finally, he achieved SVR.

Case 2

The patient was a 70-year-old woman with HCV-related liver cirrhosis and HCC. LDLT was performed in May 2006 after obtaining informed consent. Postoperatively, the patient was treated with PEG IFN-α-2b (80 μg) plus RBV (200 mg, due to anemia), which commenced in August 2006. Because serum HCV RNA titer never decreased below the detection limit (1.2 log IU/mL) in the subsequent 48 months, tacrolimus was changed to cyclosporin, and PEG IFN-α-2b plus RBV was changed to the combination of PEG IFN-α-2b (100 μg), RBV (200 mg, due to anemia) and TVR (1500 mg). At the start of triple therapy, platelet count was $19.8 \times 10^4/\mu L$, ALT was 15 IU/L, genotype was 1b, and HCV RNA was 6.2 log IU/mL. Further analysis showed no a.a. substitutions in the ISDR, but mutant- and wild-type a.a. at a.a.70 and a.a.91 in the core region, respectively were detected. The donor had TG genotype of IL-28 SNP (rs8099917) and TT/ΔG genotype of λ4 (ss469415590), while the recipient had TT genotype of IL-28 SNP (rs8099917) and TT/TT genotype of λ4 (ss469415590) (Table 2, Fig. 2). Cyclosporin was started at 10 mg/day, and based on measurement of its concentration in peripheral blood, the dose was increased gradually to 40 mg/day. Subsequent analysis showed a rise in serum creatinine and uric acid, but parameters improved following transfusion. Skin rashes of grade 2 appeared during the triple therapy, which was successfully treated with steroid cream. On the other hand, serum HCV RNA titer decreased below the detection limit (1.2 log IU/mL) at 5 weeks. However, triple therapy was stopped at 11 weeks due to general fatigue. HCV RNA rebounded 4 weeks later.

DISCUSSION

THE SVR RATE has improved since the introduction of PEG IFN/RBV for patients who undergo LT for HCV-related end-stage liver disease. The current estimated SVR rate for LT patients with history of HCV genotype 1 infection is 30–50%.[13–18] These results are much better than those reported in the 1990s and early 2000s, however, more than half of recipients still suffer from recurrent chronic hepatitis C.

It is often difficult to use protease inhibitors for HCV recipients after LT due to potential interaction with immunosuppressive drugs. We reported here our experience with two patients treated with protease inhibitors combined with PEG IFN/RBV for the treatment of recurrent post-LT hepatitis caused by genotype 1 HCV.

A recent study that examined the effect of TVR on the pharmacokinetics of cyclosporin and tacrolimus

Table 2 Laboratory data of Patient 2 at start of triple therapy after LT

CBC		LDH	241 IU/L	Tumor marker	
WBC	7530/μL	ALP	294 IU/L	AFP	5.6 ng/mL
RBC	4.23×10^6/μL	γ-GT	17 IU/L		
Hb	13.3 g/dL	TP	6.4 g/dL	HCV virus markers	
Ht	39.7%	Alb	3.5 g/dL	HCV RNA	6.2 log IU/mL
Plt	17.8×10^4/μL	TC	219 mg/dL	genotype	1b
		TTT	7 U		
Blood coagulation test		ZTT	12 U		
PT	121%	BUN	12.6 mg/dL	a.a. substitution in ISDR	0
		Cr	0.50 mg/dL	a.a.70 in the core region	Mutant
Blood chemistry		CRP	0.11 mg/dL	a.a.91 in the core region	Wild
T-Bil	0.7 mg/dL	FBS	106 mg/dL	*IL-28B* donor	TG genotype
AST	20 IU/L	HbA1c	6.9%	*IL-28B* recipient	TT genotype
ALT	15 IU/L	NH_3	57 μg/mL	ss469415590 door	TT/ΔG genotype
				ss469415590 recipient	TT/TT genotype

γ-GT, γ-glutamyltransferase; a.a. substitution in ISDR, amino acid substitutions in the interferon sensitivity-determining region; AFP, α-fetoprotein; Alb, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUC, area under curve; BUN, blood urea nitrogen; CBC, complete blood count; Cr, creatinine; CRP, C-reactive protein; FBS, fasting blood sugar; Hb, hemoglobin; HbA1c, hemoglobin A1c; Ht, hematocrit; LDH, lactate dehydrogenase; LT, liver transplantation; RBC, red blood cells; Plt, platelets; PT, prothrombin time; T-Bil, total bilirubin; TC, total cholesterol; TP, total protein; TTT, thymol turbidity test; WBC, white blood cells; ZTT, zinc sulfate turbidity test.

reported a 78-fold increase in tacrolimus blood concentration and fourfold rise in cyclosporin blood concentration through interaction with TVR.[11] For this reason, we changed tacrolimus to cyclosporin before triple therapy. We also started cyclosporin using a small dose and checked the blood concentration of cyclosporin on a daily basis. Based on these measures, cyclosporin blood concentration remained at approximately 100 ng/mL. Considered collectively, it is important to change the dose of immunosuppressive drugs and frequently monitor cyclosporin blood concentrations.

It is noteworthy that the blood concentration of TVR also increased by interaction with cyclosporin. The AUC of TVR in patient 1 was 103 μgh/mL, while the AUC of TVR of 10 chronic hepatitis C patients treated with PEG IFN/RBV was 52 μgh/mL in our hospital (data not shown). These findings highlight the need for awareness of the potential side-effects of TVR. In fact, various side-

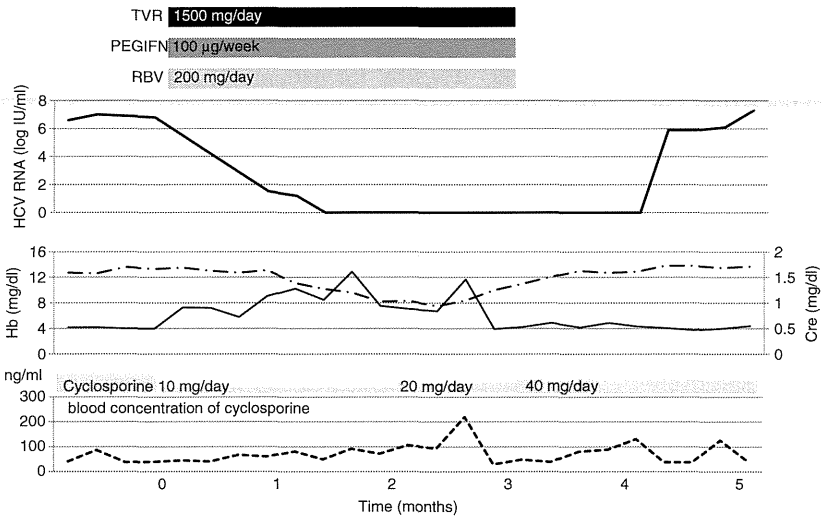

Figure 2 Clinical course of patient 2. Cre, creatinine; Hb, hemoglobin; HCV, hepatitis C virus; PEG IFN, pegylated interferon; RBV, ribavirin; TVR, telaprevir. —, Cre; — ·, Hb.

effects were reported by patient 2, including anemia, renal dysfunction and skin rashes. Consequently, the triple therapy was discontinued at 11 weeks in this patient.

What are the indications for triple therapy? While there are no standardized rules for the initiation of this mode of treatment, we believe that triply therapy should be used under the following conditions: (i) laboratory tests should show normal hemoglobin and serum creatinine levels to avoid potential side-effects of TVR; and (ii) recipients who develop HCV RNA relapse while receiving PEG IFN/RBV dual therapy after LT. In naïve cases, we recommend PEG IFN/RBV therapy. There are some reports of triple therapy for recipients after LT.[19–21] However, there is no evidence in safety of triple therapy for recipients. Furthermore, Coilly *et al.* recommends PEG IFN/RBV dual therapy for naïve cases in review.[22]

Third, both the donor and recipient must have good SNP (IL28B or λ4). On the other hand, we recommend withholding triple therapy for patients who fail to respond to PEG IFN/RBV and those who have minor SNP (IL28B or λ4) of donor and recipient. In this regard, several groups have reported that *IL28B* of both recipients and donors influenced the SVR to PEG IFN/RBV in patients with recurrent hepatitis C after LT.[23–26]T.[19–22]

Another important question regarding treatment of recurrent post-LT HCV infection is the duration of IFN therapy. The answer to this question is difficult and currently there are no data on the ideal duration of triple therapy. However, we recommend long-term PEG IFN/RBV therapy following triple therapy from 12 to 36 weeks, with a total duration of treatment of 48 weeks. This is based on our previous finding that the majority of patients with genotype 1b in whom HCV RNA reached undetectable levels were able to achieve SVR (87.5%; 7/8).[23] Eradication of HCV by triple therapy should increase the SVR rate. In fact, Pungpapong *et al.* used 12-week triple therapy followed by 36-week PEG IFN/RBV therapy and reported an SVR rate associated with this regimen of 100% (7/7) for genotype 1b recipients.[12]

On the other hand, for such hard-to-treat patients after LT, DAA will become a standard therapy in the future. Because SVR rate and safety of DAA therapy is more higher than triple therapy.[27–29] However, there is a problem of mutation of HCV against DAA therapy.[30,31] In these instances, it may be necessary to recommence triple therapy. The experience of the present study provides a good reference for such an occurrence (e.g. dose of TVR and dose of immunosuppressive agents).

In conclusion, we reported our experience with two patients who developed recurrent HCV genotype 1 infection after LT and were treated with protease inhibitors combined with PEG IFN/RBV. The results point to possible achievement of SVR by triple therapy; however, more studies are needed to evaluate the clinical benefits and side-effects of triple therapy for recurrent post-LT HCV infection.

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Attenuation of Portal Hypertension by Continuous Portal Infusion of PGE1 and Immunologic Impact in Adult-to-Adult Living-Donor Liver Transplantation

Takashi Onoe,[1,2,3] *Yuka Tanaka,*[1] *Kentaro Ide,*[1] *Kouhei Ishiyama,*[1] *Akihiko Oshita,*[1] *Tsuyoshi Kobayashi,*[1] *Hironobu Amano,*[1] *Hirotaka Tashiro,*[1] *and Hideki Ohdan*[1]

Background. Small-for-size syndrome remains the greatest limiting factor of expanding segmental liver transplantation from living donors. Portal hyperperfusion is considered to substantially contribute to small-for-size syndrome. We investigated the impact of continuous portal infusion of prostaglandin E1 (PGE1) on small-for-size grafts (SFSGs) in adult-to-adult living-donor liver transplantation (LDLT).

Methods. From July 2003 to December 2009, LDLT was performed in 122 patients. We introduced continuous portal infusion of PGE1 to five SFSG patients (PG group) from November 2007 to December 2009 and retrospectively compared them with a historical control group of eight relevant SFSG patients without PGE1 infusion (non-PG group) from July 2003 to October 2007 to determine the safety and efficacy of continuous PGE1 portal infusion for SFSGs. Splenectomy cases were excluded from analysis.

Results. The PG group demonstrated significantly lower postoperative portal pressure than the non-PG group. Moreover, the PG group demonstrated significantly improved liver function in the early posttransplantation period and significantly better recovery from hyperammonemia at 1 week after transplantation and from hyperbilirubinemia in the late posttransplantation period. Overall survival was significantly better in the PG group than in the non-PG group. Three patients in the non-PG group died of rejection-related reasons. Interestingly, immunomonitoring assay revealed that antidonor immune responses were significantly accelerated in the non-PG group compared with the PG group after LDLT. In contrast, the PG group showed well-suppressed antidonor immune responses.

Conclusion. Continuous portal infusion of PGE1 for SFSG attenuated portal hypertension, improved graft function, and suppressed antidonor immune responses, resulting in better survival.

Keywords: Living-donor liver transplantation, Small-for-size graft, Portal hypertension, Alloimmune response, Prostaglandin E1.

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[1] Department of Gastroenterological and Transplant Surgery, Applied Life Sciences, Institute of Biomedical & Health Sciences, Hiroshima University, Hiroshima, Japan.

[2] Department of Clinical Research, National Hospital Organization Kure Medical Center/Chugoku Cancer Center, Hiroshima, Japan.

[3] Address correspondence to Takashi Onoe, M.D., Ph.D., Department of Gastroenterological and Transplant Surgery, Applied Life Sciences, Institute of Biomedical & Health Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima City, Hiroshima 734-8551, Japan.

E-mail: tonoemd@gmail.com

T.O. and H.O. participated in the research design, performance of the research, and writing of the article. Y.T. participated in data analysis. K.I., K.I., A.O., T.K., H.A., and H.T. participated in the performance of the research.

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Segmental liver transplantation based on cadaveric splitting or living-donor liver transplantation (LDLT) has been developed for treating patients with end-stage liver disease. It is also a means of overcoming organ shortage and wait-list mortality. However, small-for-size syndrome (SFSS) remains the greatest limiting factor for the expansion of segmental liver transplantation from either cadaveric or living donors (*1, 2*). If the volume of the engrafted liver is considerably less than the standard liver weight in patients with end-stage liver disease who are undergoing partial liver transplantation, excessive portal venous inflow might cause early portal hypertension (*3, 4*) and increased morbidity and mortality due to SFSS (*5*). Previous data have suggested that, in recipients of adult-to-adult LDLT, one of the most challenging tasks is to match a good size graft. Emphasis has more recently been placed not only on the evaluation of the ratio between donor and recipient liver volume but also on the degree of portal hypertension and the stage of liver disease in the recipient, consistent with the result in a pig model (*6–8*). Therefore, the importance of portal pressure during LDLT is now recognized.

We have demonstrated that continuous portal infusion of prostaglandin E1 (PGE1) considerably improved the congestion

of the residual liver after extended hepatectomy in a rat model (*9*). Based on this result, we applied a continuous portal infusion of PGE1 for small-for-size grafts (SFSGs) in LDLT in the clinical setting.

We here investigated the clinical significance of controlling portal pressure by continuous portal infusion of PGE1 after surgery in LDLT with SFSGs, focusing on portal decompression, postoperative liver function, survival, and the antidonor immune status of the recipient retrospectively.

RESULTS

Patients' Demographic and Clinical Characteristics

Thirteen patients receiving SFSGs were retrospectively analyzed in this study. The patients' demographic and clinical characteristics are shown in Table 1. Of these patients, five received a continuous portal injection of PGE1 after transplantation (PG group) from November 2007 to December 2009 (era 2), whereas eight were historical controls from July 2003 to October 2007 (era 1) without PGE1 infusion (non-PG group). There was no significant difference in age or underlying disease between the two groups. Preoperative examination of the hepatic reserve showed similar Child-Pugh scores (PG group, 10.0±0.71; non-PG group, 9.00±0.83). Patients' model for end-stage liver disease scores, which were used as recipient severity indices, was similar between groups (mean [range], 16.8 [8–30] and 15.1 [9–28], respectively). Portal vein pressure (PVP) at laparotomy was also similar between the two groups (25.2 [17–34] and 20.3 [17–24] mm Hg, respectively). Concerning the graft, one patient in each group showed minimal fatty metamorphosis (<0.1%) on histology and there was no significant difference in graft-to-recipient body weight ratio (GRWR) between the two groups (0.680 [0.63–0.71] and 0.655 [0.51–0.72], respectively).

Furthermore, factors related to surgical invasiveness in those two groups, such as hemorrhage level, operation time, and graft ischemia duration, were similar. No donor had donor-specific antigens, and there was no difference in the number of human leukocyte antigen (HLA) mismatch (Table 1). Three donor candidates in each group underwent liver biopsy. Among them, one in each group showed minimal fatty metamorphosis (<0.1%) on histology. Of note, three of five patients in the PGE1 group and three of eight patients in the non-PGE1 group received right-lobe grafts. All patients receiving right lobes in both groups had grafts with middle hepatic vein (MHV) tributaries more than 5 mm in diameter, and all draining tributaries were reconstructed with the

TABLE 1. Patients' demographic and clinical characteristics

Variables	PG group (n=5)	Non-PG group (n=8)	*P*
Recipient factors			
Age, years	56.4±3.4	57.9±4.4	0.510[a]
Gender, male/female	5/0	3/5	0.075[b]
Child-Pugh score	10.0±1.6	9.0±1.9	0.325[a]
MELD score	16.8±8.2	15.1±5.8	0.702[a]
PVP, mm Hg, at laparotomy	25.2±6.1	20.9±3.0	0.199[a]
Disease background			
Viral hepatitis (B/C)	1/2	1/5	>0.999[b]
Alcoholic	1	1	>0.999[b]
Acute hepatic failure	1	0	0.385[b]
Cholestatic disease	0	1	>0.999[b]
Donor factors			
Age, years	26.2±3.3	33.3±10.5	0.113[a]
Gender, male/female	0/5	5/3	0.075[b]
Graft factors			
Graft type, right/left	3/2	3/5	0.592[b]
GRWR, %	0.68±0.03	0.66±0.09	0.510[a]
Reconstruction of hepatic vein	3	3	0.592[b]
HLA class I mismatch	1.20±0.49	1.63±0.23	0.453[a]
HLA class II mismatch	0.60±0.24	1.00±0.00	—
DSA	0	0	—
Surgical factors			
Operation time, min	781.0±153.6	755.9±106.0	0.758[a]
Bleeding, mL	5322.0±2295.3	5751.4±6371.2	0.866[a]
Total ischemia time, min	117.0±35.5	118.9±31.4	0.925[a]

[a] Unpaired *t* test with Welch's correction.
[b] Fisher's exact test.
DSA, donor-specific antibody; HLA, human leukocyte antigen; GRWR, graft-to-recipient body weight ratio; MELD, model for end-stage liver disease; PVP, portal vein pressure.

recipients' native MHV trunk as reported previously (*10*). There was no thrombosis in those reconstructed tributaries after surgery. One patient of each group had grafts with inferior right hepatic vein, which were reconstructed using direct anastomosis to inferior vena cava in each case.

Continuous PGE1 Infusion Attenuated Portal Hypertension After Reperfusion in SFSGs

After laparotomy, we inserted a catheter from the mesenteric vein to the distal side of the portal vein and measured the PVP during the operation. All patients exhibited portal hypertension during laparotomy. In the PG group, after reflow of the portal and hepatic veins was confirmed, we started PGE1 infusion into the portal vein through a catheter. Continuous infusion of PGE1 resulted in a significant reduction of PVP at the time of abdominal closure in the PG group compared with the non-PG group ($P<0.005$; Fig. 1A). The mean PVP at the time of abdominal closure was 15.4±1.17 mm Hg in the PG group and 20.5±1.47 mm Hg in the non-PG group (Fig. 1A). Furthermore, the PVP ratio at the end of the operation, compared with that at laparotomy, showed effective portal decompression in the PG group and non-PG group, respectively (0.62±0.04 vs. 0.99±0.06; $P<0.001$; Fig. 1B). Importantly, none of the patients in the PG group developed hypoperfusion after PGE1 portal infusion.

Clinical Course of Graft Liver Function

Graft liver function markers, including serum transaminases, arterial ketone body ratio (AKBR), ammonia, and total bilirubin, after surgery were compared between the PG group and the non-PG group.

Elevated serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were significantly attenuated in the PG group compared with the non-PG group on days 1 and 2 (Fig. 2). Similarly, the AKBR, which reflects the hepatic mitochondrial redox state and is considered an accurate index of the functional reserve of the graft liver after transplantation, was significantly higher in the PG group. However, these values became comparable between the two groups after day 3. Strikingly, significantly better recovery from hyperammonemia was seen in the PG group for 1 week after surgery. The serum total bilirubin level was comparable between the two groups by day 28 after LDLT. Nonetheless, hyperbilirubinemia was significantly improved in the PG group after day 28 but remained prolonged in the non-PG group. These results indicate that continuous infusion of PGE1 significantly improved the liver function after LDLT with SFSGs.

Complications and Prognosis

In the PG group, no complications associated with the portal vein catheter were observed after surgery (e.g., post-removal bleeding, catheter infection, or portal thrombosis). One patient in the non-PG group and none in the PG group developed SFSS. Postoperative death occurred in 5 patients of the non-PG group and in none in the PG group. In the non-PG group, the 1- and 2-year survival rates were 62.5% and 37.5%, respectively. In contrast, in the PG group, the 1-and 2-year survival rates were both 100%, a difference that was statistically significant ($P<0.05$; Fig. 3). The main causes of death in the non-PG group were graft dysfunction, rejection, and subsequent infection as well as bacterial sepsis after biliary stenosis. No patients in the PG group had a

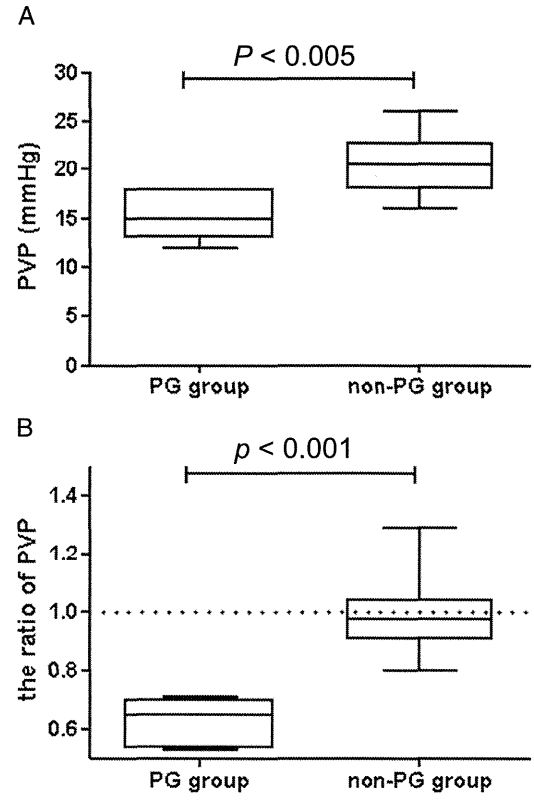

FIGURE 1. PVP value at the end of the operation (A) and ratio of PVP at the end of the operation to that at laparotomy (B) in the PG group and the non-PG group. An unpaired *t* test with Welch's correction was used to compare PVP and the ratio of PVP between the PG group and the non-PG group. The box plot represents the 25th to 75th percentiles, the dark line is the median, and the extended bars represent the 10th to the 90th percentiles. *$P<0.05$; ***$P<0.001$. PVP, portal vein pressure.

rejection episode. Rejection was diagnosed by liver biopsy and histologic findings showed features of SFSG and/or portal hypertension with rejection (see **Figure S1, SDC**, http://links.lww.com/TP/A807). The 2-year survival of SFSG patients (non-PG group) in era 1 (July 2003 to October 2007) was significantly worse than that of the non-SFSG patients in the same period (37.5% vs. 77.8%; $P<0.05$), whereas the 2-year survival of SFSG patients (PG group) in era 2 (November 2007 to December 2009) was not statistically different from that of the non-SFSG patients in the same period (100% vs. 77.1%). Of note, the 2-year survival of non-SFSG patients was similar between eras 1 and 2 (Fig. 4).

Estimation of Immunosuppressive Status After Surgery by Using the Carboxyfluorescein Diacetate Succinimidyl Ester-Mixed Lymphocyte Reaction Assay

Because the main cause of death in 3 patients in the non-PG group was related to rejection, we retrospectively analyzed the immunosuppressive postoperative status of both groups. All patients and their donors consented to be

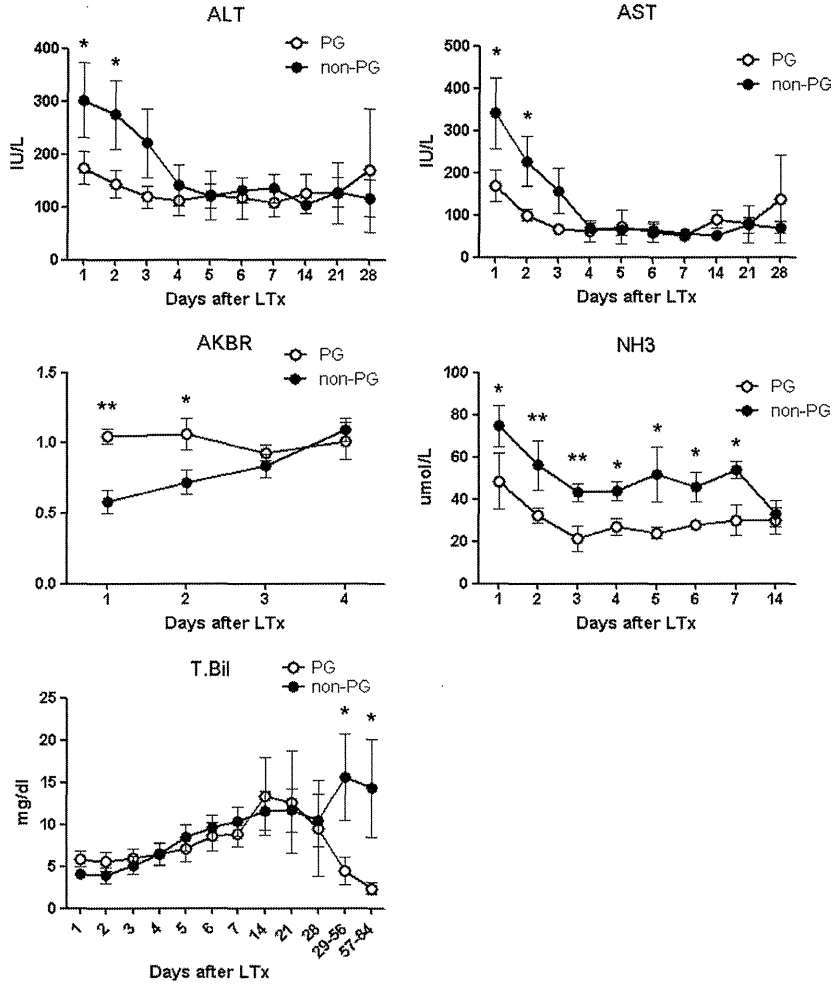

FIGURE 2. Liver function tests (ALT, AST, AKBR, NH_3, and T.Bil) of patients with (PG group; open circle) or without PGE1 portal infusion (non-PG group; closed circle) after LDLT. Data are mean±SEM for individual groups. An unpaired *t* test with Welch's correction was used to compare each of the indicated parameters between the PG group and the non-PG group. **P*<0.05; ****P*<0.01. AKBR, arterial ketone body ratio; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDLT, living-donor liver transplantation; LTx, liver transplantation; NH_3, ammonia; T.Bil, total bilirubin.

subjected to a mixed lymphocyte reaction (MLR) assay with the carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling technique. In all five patients of the PG group, suppressed $CD8^+$ T-cell proliferation, which is defined as a stimulation index (SI)<2, was observed in the antidonor MLR assay (i.e., a hyporesponse to donor; mean SI, 1.10±0.13; Fig. 4A). The mean percentage of $CD25^+$ cells among the proliferating $CD8^+$ T cells, which are activated cytotoxic T cells, was 9.24±5.93 (Fig. 4B). In contrast, in five of the eight patients in the non-PG group, accelerated $CD8^+$ T-cell proliferation was observed in the antidonor MLR assay (i.e., a hyperresponse to donor; mean SI, 2.85±0.50; Fig. 4A). Furthermore, the mean percentage of $CD25^+$ cells among the proliferating $CD8^+$ T cells was 63.82±8.63 (Fig. 4B). These differences between the two groups were significant. Of note, three patients in the non-PG group who showed high antidonor response (i.e., SI of $CD8^+$ T cells>3) required steroid pulse treatment and died of graft dysfunction or infection after rejection. Two patients who showed a relatively high antidonor response (i.e., SI of $CD8^+$ T cells>2) required an increase in immunosuppressant doses. These results indicated that patients with SFSGs show accelerated antidonor immune responses and that continuous portal infusion of PGE1 suppressed this type of antidonor immune response.

DISCUSSION

Various approaches to controlling excessive portal flow and pressure have been proposed, such as dual grafting to increase graft volume (*11, 12*). Although this concept is simple, it requires two healthy living donors and involves increased risk to donors. Another approach is portal decompression with a portosystemic shunt (*13, 14*) or splenic artery manipulation, including splenectomy, embolization, and ligation (*15–17*). This method is more favored in terms of availability and donor risk. Nonetheless, there is

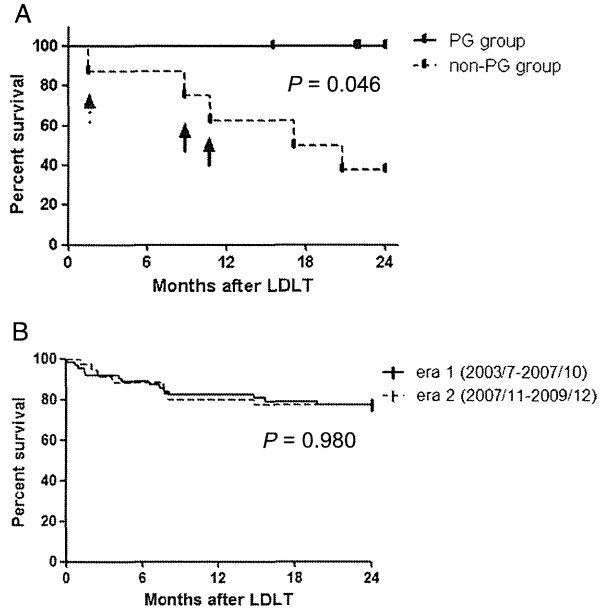

FIGURE 3. A, Kaplan–Meier patient survival curves of patients with (PG group; n=5; solid line) or without PGE1 portal infusion after LDLT (non-PG group; n=8; dotted line). In the non-PG group, the 1- and 2-year survival rates were 62.5% and 37.5%, respectively. In the PG group, the 1- and 2-year survival rates were both 100%, a difference that was statistically significant. *P<0.05. Dashed arrow represents a patient's death due to SFSS and rejection followed by infection, and solid arrows represent patients' death due to rejection-related reasons. B, Kaplan–Meier patient survival curves of non-SFSG patients in era 1 (from July 2003 to October 2007; n=62; solid line) or era 2 (from November 2007 to December 2009; n=35; dotted line). In the era 1 and era 2 groups, the 2-year survival rate was 77.4% and 77.1%, respectively, with no statistical difference (P=0.980). ABO-incompatible cases and splenectomy cases were excluded from analysis. LDLT, living-donor liver transplantation; PGE1, prostaglandin E1; SFSG, small-for-size graft; SFSS, small-for-size syndrome.

a considerable risk of infection in splenectomy or splenic artery ligation (*18*). Moreover, significantly higher mortality was observed in patients who had splenectomy mainly due to septic complications in liver transplantation (*19, 20*). In fact, we experienced one SFSG case in which the patient died of sudden sepsis without any primary focus 4 years after transplantation with splenectomy. Therefore, another method to control portal pressure and preserve the spleen is likely more preferable.

We have reported that portal administration of PGE1, a vasodilator of vessels containing smooth muscle (*21, 22*), prevented congestion of residual liver tissues in a rat extended hepatectomy model. In this study, we tried various vasodilators; however, residual liver congestion after hepatectomy was improved only by continuous portal infusion of PGE1. We also tried systemic continuous venous infusion of PGE1 at the same dose, but this was not effective. This suggests the therapeutic potential of portal PGE1 injection to prevent portal hypertension after LDLT with SFSGs.

We translated this method to adult LDLT with SFSGs, and portal infusion of PGE1 successfully reduced PVP, resulting in improved liver graft function in both early and late posttransplantation periods. This result was unexpected because the portal infusion of PGE1 was given for only the first week yet improved the long-term survival of recipients.

We used a CFSE-MLR assay to objectively evaluate the antidonor responses of the recipients (*23, 24*). The lack of $CD8^+$ and $CD25^+$ T-cell proliferation in antidonor MLR reflects the suppression of the antidonor response. In this immunologic investigation, all patients given the continuous portal infusion of PGE1 showed a well-suppressed response of the antidonor $CD8^+$ T cells (Fig. 4). In contrast, surprisingly, patients without the PGE1 treatment showed

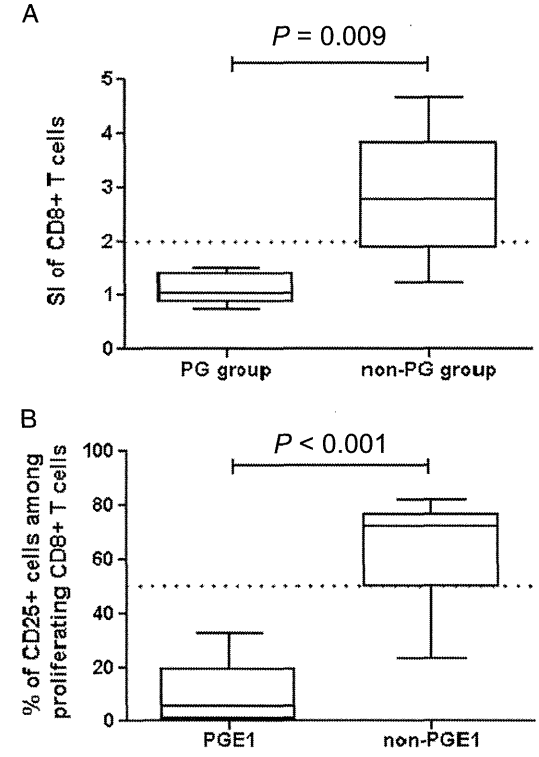

FIGURE 4. SIs of $CD8^+$ T-cell subsets in the antidonor MLR assay of patients in the PG group (n=5) and the non-PG group (n=8) on the third to fourth weeks after transplantation (A) and percentage of $CD25^+$ cells among proliferating $CD8^+$ T cells in patients of the PG group and the non-PG group (B). $CD8^+$ T-cell proliferation and their SIs were quantified as follows. The number of division precursors was extrapolated from the number of daughter cells of each division, and the number of mitotic events in each of the $CD4^+$ and $CD8^+$ T-cell subsets was calculated. Using these values, the mitotic index was calculated by dividing the total number of mitotic events by the total number of precursors. The SIs of the allogeneic combinations were calculated by dividing the mitotic index of a particular allogeneic (self to donor) combination by that of the self-control. An unpaired t test with Welch's correction was used to compare the SI and percentage of $CD25^+$ cells between the PG group and the non-PG group. The box plot represents the 25th to 75th percentiles, the dark line is the median, and the extended bars represent the 10th to the 90th percentiles. **P<0.01; ***P<0.001. MLR, mixed lymphocyte reaction; SI, stimulation index.

an accelerated response of antidonor $CD8^+$ T cells despite the use of the same immunosuppressive protocol. Therefore, SFSG likely accelerated the antidonor response, enhanced rejection, and might result in a worse survival rate, although SFSS is multifactorial in nature. This finding is consistent with the results in an animal model (*25*) and our preliminary data using mouse model (data not shown). To our knowledge, this is the first clinical report to state that SFSG may accelerate antidonor immune responses in LDLT.

We previously reported that liver sinusoidal endothelial cells (LSECs) of grafts induce allospecific immunotolerance by suppressing reactive T cells through Fas ligand and/or PD-L1 signaling (*26–28*). Based on this concept, one possible mechanism is that the sinusoidal structure and LSECs could be damaged by portal hypertension and lose their tolerogenicity, resulting in accelerated antidonor immunoresponse and rejection. This interpretation is consistent with that of previous reports stating that portal hypertension disrupts sinusoids and LSECs in the liver (*8*). Another possibility is antirejection and/or the anti-inflammatory effect of PGE1 itself. It has been reported that PGE1 could prevent ischemia-reperfusion injury by inducing heat shock protein (*29*) or by inhibition of neutrophil adherence (*30*). It has been also reported that administration of PGE1 could prevent and suppress the rejection process in heart transplantation (*31*) and in renal transplantation (*32*). Furthermore, it has been reported that PGE1 protects human LSECs from apoptosis, which is consistent with our findings (*33*).

The main limitation of our study is its retrospective nature. Another limitation is the relatively small number of patients in each subgroup analyzed, although the background characteristics of each group and the survival rates of non-SFSG patients were similar. A randomized study is ideal; however, performing a prospective randomized study for this approach is difficult because of the high mortality of SFSG patients without PGE1 perfusion, as shown in Results.

In conclusion, continuous infusion of PGE1 is suggested to be useful in improving SFSG function and survival after LDLT. Improved understanding of underlying mechanisms may have important implications for clinical managements such as antirejection therapy or preventing ischemia-reperfusion injury in liver transplantation with SFSGs.

MATERIALS AND METHODS

Patients

From July 2003 to December 2009, LDLT was performed on 122 adult patients with end-stage liver disease. We introduced continuous portal infusion of PGE1 to five patients with SFSGs that exhibited a GRWR less than 0.72% from November 2007 to December 2009 (era 2). These patients (PG group) were the subjects of this retrospective case–control study. We compared them with a historical group of eight relevant patients who received SFSG without PGE1 infusion (non-PG group) from July 2003 to October 2007 (era 1) to determine the safety and efficacy of continuous PGE1 portal infusion for SFSGs. Because we introduced the portal infusion of PGE1 in November 2007, all patients with SFSGs in era 2 received PGE1 infusion. One SFSG case with splenectomy in era 1 was excluded from this study. The study protocol was approved by the ethics committee of Hiroshima University, and all patients provided informed consent before surgery. None of the patients receiving PGE1 portal infusion showed clinical evidence of insertion site infection or bleeding after catheter removal throughout the follow-up period.

Operation, PVP Measurement, and Continuous Portal Infusion of PGE1

The graft-harvesting technique, recipient surgery, perioperative recipient management, and immunosuppression regimens were conducted as described previously with minor modifications (*34, 35*). In brief, the right lobe without the MHV or the left lobe with the MHV was harvested from the donor as follows. Before parenchymal transection, the right or left lobe was mobilized and the short hepatic veins were transected. For the right lobe, during parenchymal transection, the major right tributaries of the MHV were clamped using a vascular clip and then transected. After hepatectomy, ex vivo perfusion of the graft was performed through the portal vein. The initial perfusate was saline solution (500 mL); then, the University of Wisconsin solution (1000 mL) was used as the perfusate.

To measure the PVP in the recipient during the operation, an 18G catheter was inserted from the mesenteric vein to the portal vein after laparotomy (*36*). The implantation was performed after total hepatectomy. The graft vein was anastomosed to the equivalent vein of the recipient in an end-to-end fashion. Thereafter, the graft was reperfused before microsurgical reconstruction of the hepatic artery (end-to-end anastomosis of the graft hepatic artery to the recipient hepatic artery). The bile duct of the graft liver was anastomosed in an end-to-end fashion to the recipient's common hepatic bile duct. In the non-PG group, the portal catheter was removed at the time of abdominal closure. In the PG group, PGE1 was administered through a portal catheter at the graft portal reflow. PGE1 was continuously administered for 1 week ($0.01\ g\ kg^{-1}\ min^{-1}$) and then the catheter was noninvasively removed. AST, ALT, serum bilirubin, serum ammonia, and AKBR levels were measured as liver function indices. The initial immunosuppressive regimen consisted of tacrolimus and steroids. Doppler ultrasonography and computed tomographic scans were routinely performed daily and biweekly, respectively.

Immunosuppression was initiated with a protocol based on tacrolimus (Prograf; Astellas Pharma, Tokyo, Japan) and methylprednisolone. Methylprednisolone was withdrawn gradually according to the protocol. The dose of tacrolimus was controlled according to blood concentration and adjusted daily. The target trough level was set at 15 ng/mL for 2 weeks and 10 ng/mL for another 2 weeks. Continuous venous infusion of heparin for therapeutic heparinization was routinely done to prevent thrombosis, which was monitored using coagulation tests. Rejection was diagnosed and proven by biopsy histologically and MLR assay. Patients were followed for 2 years after LDLT, and survival was defined as the period between LDLT and death.

Immune Monitoring by In Vitro CFSE-MLR Assay

CFSE-MLR was routinely performed to evaluate the recipient's antidonor immune response 2 to 4 weeks after surgery.

For CFSE-MLR, peripheral blood mononuclear cells prepared from the blood of the recipients (autologous control), donors, and healthy volunteers with the same blood type as the donors (third-party control) for use as the stimulator cells were irradiated with 30 Gy. Those obtained from the recipients for use as the responder cells were labeled with 5 mM CFSE (Molecular Probes, Eugene, OR), as described previously (*24*). The stimulator and responder cells (2×10^6 each) were incubated in 24-well flat-bottomed plates in a total volume of 2 mL culture medium at 37°C under 5% CO_2 for 5 days. After culture for MLR, $CD4^+$ and $CD8^+$ T-cell proliferation, CD25 expression of proliferating T-cell subsets and SI were quantified by flow cytometry as described previously (*37, 38*) and described in the SDC Materials and Methods in detail (see http://links.lww.com/TP/A807).

Statistical Analysis

Statistical analysis and comparisons were performed using PRISM version 4.0 (GraphPad, San Diego, CA). Data are expressed as mean±SEM. An unpaired *t* test with Welch's correction was used to compare groups. *P* values <0.05 were considered statistically significant.

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INVITED COMMENTARY

Is living donor liver transplantation really equivalent to deceased donor liver transplantation?*

Hideki Ohdan

Department of Gastroenterological and Transplant Surgery, Applied Life Science, Institute of Biomedical and Health Science, Hiroshima University, Hiroshima, Japan

Correspondence
Hideki Ohdan MD, PhD,
Department of Gastroenterological and Transplant Surgery, Applied Life Science, Institute of Biomedical and Health Science, Hiroshima University 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan.
Tel.: +81-82-257-5220;
fax: +81-82-257-5224;
e-mail: hohdan@hiroshima-u.ac.jp

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*Invited commentary on "Living donor versus deceased donor liver transplantation: A surgeon-matched comparison of recipient morbidity and outcomes", by Reichman *et al.*

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Living donor liver transplantation (LDLT) has developed as an alternative to deceased donor liver transplantation (DDLT) in order to overcome the critical shortage of deceased organ donations. Particularly in regions with low deceased donation rates, like Asian, LDLT for end stage liver disease significantly reduces the risk of death or drop off the wait list without compromising post-transplant survival. A preference for LDLT to DDLT may depend on the original disease representing the indication for liver transplantation (LT). LDLT offers a timely alternative to DDLT for patients with hepatocellular carcinoma (HCC). However, the higher recurrence rate of HCC after LDLT and the indication criteria remain controversial. One of the recent quantitative meta-analyses revealed the comparable patient survival rates and no significant differences in the recurrence rates between LDLT and DDLT recipients [1]. Another meta-analysis provided evidence of lower disease-free survival (DFS) after LDLT compared with DDLT for HCC [2]. Hence, LDLT likely represents an acceptable option that does not compromise patient survival or increase HCC recurrence in comparison with DDLT at this moment.

Early data suggested that patients with Hepatitis C virus (HCV) that received a LDLT had worse outcomes, including increased rates of cholestatic HCV than did recipients of DDLT [3,4]. This is currently thought to be because of an increased rate of biliary complications or other problems seen during the learning curve of early LDLT experience. More recent data demonstrated that there is no difference in recurrent HCV between recipients of DDLT and LDLT [5,6]. The latest meta-analysis demonstrated that LDLT was equivalent to DDLT in terms of long-term patient or graft survival, HCV recurrence, and acute rejection with a potential lower short-term graft survival [7].

There are limited convincing data comparing outcomes of LDLT and DDLT for autoimmune hepatitis (AIH) and cholestatic liver diseases. It has been previously reported that the overall survival outcomes of LDLT were similar to DDLT in patients with AIH and primary biliary cirrhosis

[8]. In contrast, patients with primary sclerosing cholangitis undergoing LDLT, especially with biologically related donors, are thought to have a higher risk to develop recurrent disease compared with the DDLT setting, probably because of sharing antigens targeted by autoimmunity between recipients and the related donors [9]. Further prospective studies at transplant centers performing both LDLT and DDLT might be needed to confirm these issues.

Regardless of such original disease, LDLT offers several advantages over DDLT, which include the reduction in waiting time mortality, the reduction in cold ischemic time (CIT) and the feasibility of various preoperative interventions, such as nutritional treatment for both the donor and recipient [10]. However, it remains unclear whether those advantages offset disadvantages peculiar to LDLT, such as the smaller graft volume than DDLT and the highly technical procedure, which may be associated with higher complication rates. This seems to be caused by a fact that direct comparison of the results between LDLT and DDLT inevitably involves various biases in nature.

Reichman *et al.* [11] have performed a retrospective matched-cohort study to compare postoperative complication rate and patient survival in the two groups of patients submitted to LDLT and to DDLT. Six clinical variables for recipients: age, Meld, date of transplant, gender, primary diagnosis, and recipient surgeon were matched in each group (n = 145 in each group). They found that the overall complication rate was similar between two groups. In further detail, biliary complications were higher in LDLT although the complications that occurred in the DDLT were strongly associated with graft loss. Graft and patient survival outcomes for LDLT versus DDLT were similar. From those findings, they concluded that LDLT offers an excellent alternative to DDLT in areas of deceased donor organ shortages. This study defined surgical complications that are more frequent in LDLT, i.e., biliary complications (34% and 17% in LDLT and DDLT cohorts, respectively). Despite a higher rate of complications among LDLT recipients, complications leading to death were not significantly higher in LDLT in the experienced center. These findings, in concert with the current common consent that the incidence of complications, even biliary complications, can decline with center experience to levels comparable with DDLT [12], underscore the impact of the learning curve on this highly technical procedure. Potential recipients need to hear about both the rates of complications after LDLT and DDLT, and this study with control for recipient variables will help to define those rates. As pointed out by the authors, this study left control for donor variables out of consideration, despite a well known fact that donor age/gender and donor-recipient human leukocyte antigen matching correlate with either the incidence of certain complications or the severity of original disease recurrence. Nevertheless, this case control comparison of the outcome of LDLT and DDLT convincingly reported that these procedures had different complication profiles but the overall outcomes were similar with expert management, suggesting that the biological advantage in LDLT could compensate for a higher rate of surgical complications caused by greater technical complexity.

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ELSEVIER

Comparative Analysis of T-Cell Depletion Method for Clinical Immunotherapy—Anti-Hepatitis C Effects of Natural Killer Cells Via Interferon-γ Production

M. Ohira, S. Nishida, T. Matsuura, I. Muraoka, P. Tryphonopoulos, J. Fan, A. Tekin, G. Selvaggi, D. Levi, P. Ruiz, C. Ricordi, H. Ohdan, and A.G. Tzakis

ABSTRACT

Liver transplantation (LT) is a life-saving treatment for liver cirrhosis patients with hepatocellular carcinoma (HCC). However, 10%–20% HCC recurrence rate after LT is due to the immunosuppression inducing tumor growth. We recently reported a novel immunotherapy with donor liver natural killer (NK) cells to prevent HCC and hepatitis C virus (HCV) recurrence after LT. In this cell processing procedure, Muromonab-CD3 (Orthoclone OKT3, an anti-CD3 antibody) was added to the culture medium to deplete $CD3^+$ T cells to prevent graft-versus-host disease. However, the manufacture of OKT3 was discontinued in 2010, when other treatments with similar efficacy and fewer side effects became available. In this study, we examined alternative reagents for T-cell depletion-MACS GMP CD3 pure (GMP CD3), antithymocyte globulin, and alemtuzumab-for NK cell immunotherapy in the allogeneic setting. We observed that GMP CD3 showed exactly the same effects on liver mononuclear cells as OKT3, including activation of NK cells and depletion of T cells. Interestingly, binding of T-cell depletion antibodies to NK cells led to an anti-HCV effect via interferon-γ production. These results with the use of in vitro culture systems suggested that antibodies which produce T-cell depletion affected NK cell function.

Liver failure and hepatocellular carcinoma (HCC) caused by chronic hepatitis C virus (HCV) infection are the most common indications for liver transplantation (LT). The incidences of both conditions have been projected to increase further. On the one hand, the rate of HCC recurrence after LT is 10%–20%.[1,2] On the other hand, recurrent HCV infection in the allograft, which is universal, occurs immediately after LT and is associated with accelerated progression to liver cirrhosis, graft loss, and death.[3,4] These recurrences remains the most serious issue with LT. The use of postoperative immunosuppressants poses an additional risk for recurrences and hinders the use of chemotherapeutic or interferon (IFN) agents.[5,6] However, no definitive treatment or prevention for HCC recurrence after LT is known.

Natural killer (NK) cells are innate immune lymphocytes that are identified by their expression of the CD56 surface antigen and the absence of CD3 markers.[7,8] NK cells can directly kill targets through the release of granzymes, which are granules containing perforin and serine proteases, and/or by surface-expressed ligands that engage and activate death receptors expressed on target cells. Unlike T cells, NK cells do not require the presence of a specific antigen to kill cancer cells, modified cells, or invading infectious microbes. NK cells are abundant in the liver, in

From Department of Gastroenterologic and Transplant Surgery (H.O.), Applied Life Sciences, Institute of Biomedical & Health Sciences, Hiroshima University, Hiroshima, Japan; Division of Liver and Gastrointestinal Transplantation, (M.O., S.N., T.M., I.M., P.T., J.F., A.T., G.S., D.L.), Department of Surgery, University of Miami Miller School of Medicine, Miami, Florida; Department of Pathology and Surgery (P.R.), University of Miami Miller School of Medicine, Miami, Florida; Cell Transplant Center, (C.R.) Diabetes Research Institute, University of Miami Miller School of Medicine, Miami, Florida; Division of Liver and Gastrointestinal Transplantation (A.G.T.), Department of Surgery, University of Miami Miller School of Medicine, Miami, Florida; Department of Surgery, Cleveland Clinic, Weston, Florida.

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Address reprint requests to Seigo Nishida, MD, PhD, Division of Liver and Gastrointestinal Transplantation, Department of Surgery, University of Miami Miller School of Medicine, 1801 NW 9th Avenue, Miami, FL 33136. E-mail: snishida@med.miami.edu

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contrast to their relatively small distribution in peripheral lymph and lymphatic organs in rodents[9–11] and humans.[12,13] In addition, hepatic NK cells in humans have been shown to mediate cytotoxic activity against HCC[12] and to display anti-HCV effects[14] compared with their peripheral blood counterparts. We have successfully applied adoptive immunotherapy with liver NK cells to LT recipients with HCC in Japan and the United States.[14–16] In this regimen, LT recipients are injected intravenously with interleukin (IL) 2–activated NK cells derived from the donor liver allograft. After treatment with IL-2 and OKT3 (Orthoclone OKT3, an anti-CD3 monoclonal antibody [mAb]; Ortho Biotech, Raritan, NJ), liver NK cells expressed significantly elevated levels of the tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), a crucial molecule for killing of tumor cells. Furthermore, these cells showed great cytotoxicity against HCC without any effect on normal cells.[12]

OKT3, a potent immunosuppressant, has been shown to reverse renal allograft rejection episodes.[17,18] It has also been widely used for immunotherapy, as well as to expand cytotoxic T cells[19] and enhance the activity of lymphokine-activated killer (LAK) cells,[20–25] and prevent graft-versus-host disease (GVHD).[26–29] In the latter setting, administration of OKT3-coated T cells in vivo opsonizes for the reticuloendothelial system to subsequently trap or lyses cells.[30–32] This method has been used for clinical NK therapy in Japan, achieving protection against GVHD.[14] However, because of its numerous side effects, the availability of better-tolerated alternatives, and its declining use, OKT3 has been recently removed from the market. Therefore, alternative reagents need to be evaluated for this immunotherapy. In the present study, we evaluated the effect of alternative reagents-GMP CD3 (MACS GMP CD3 pure; Miltenyi Biotec, Bergisch Gladbach, Germany), antithymocyte globulin (Thymoglobulin; Genzyme, Cambridge, MA), and alemtuzumab (Campath; Genzyme) using culture systems with NK and T cells for subsequent application in clinical trials.

MATERIALS AND METHODS

Isolation of Liver Mononuclear Cells

Liver mononuclear cells (LMNCs) from liver perfusates were isolated by gradient centrifugation with Ficoll-Hypaque (GE Healthcare, Pittsburgh, PA) before suspension in X-Vivo 15 medium (Lonza, Walkersville, MD) supplemented with 100 μg/mL gentamicin (APP Pharmaceuticals, Schaumburg, IL), 10% human AB serum (Valley Biomedical, Winchester, VA), and 10 U/mL sodium heparin (APP Pharmaceuticals), as previously described.[16] Our Institutional Review Board (IRB) approved this study.

Cell Culture

LMNCs were cultured with 1,000 U/mL human recombinant IL-2 (Proleukin; Novartis, Emeryville, CA) in culture medium at 37°C in an atmosphere supplemented with 5% CO_2. LMNCs were exposed to a OKT3 (1 μg/mL), GMP CD3 (1 μg/mL), antithymocyte globulin (100 μg/mL), or alemtuzumab (100 μg/mL) at 1 day before cell harvest. After 4 days of culture, cells were subjected to further analyses.

Flow Cytometry

All flow cytometry (FCM) analyses were performed on an LSR II Flow Cytometer (BD Biosciences, San Jose, CA). The following mAbs were used for surface staining of the lymphocytes: fluorescein isothiocyanate–conjugated anti-CD3 (HIT3a; BD Pharmingen, San Diego, CA) or anti-CD56 (B159; BD Pharmingen); phycoerythrin (PE)-conjugated anti-TRAIL (RIK-2; BD Pharmingen), anti-NKp44 (P44-8.1; BD Pharmingen), or anti-CD158b (CH-L; BD Pharmingen); allophycocyanin (APC)–conjugated anti-CD56 (B159; BD Pharmingen), anti-CD25 (M-A251; BD Pharmingen), or anti-NKG2A (Z199; Beckman Coulter, Fullerton, CA); APC-eFluor780–conjugated anti-CD3 (UCHT1; eBioscience, San Diego, CA); PE-Cy7–conjugated anti-CD69 (FN50; Biolegend, San Diego, CA), or anti-NKG2D (1D11; Biolegend); eFluor 605NC–conjugated anti-CD16 (eBioCB16; eBioscience); Alexa Fluor 647–conjugated anti-NKp30 (P30-15; Biolegend); peridinin chlorophyll protein complex (PerCP)-Cy5.5–conjugated anti-CD158a (HP-MA4; eBioscience); and biotin-conjugated anti-CD122 (Mik-b3; BD Pharmingen), anti-NKp46 (9E2; Biolegend), or CD132 (TuGh4; BD Pharmingen). The biotinylated mAbs were visualized with the use of PerCP-Cy5.5-streptavidin (eBioscience) or PE-Cy7-streptavidin (Biolegend). Dead cells were excluded by light scatter and 4′,6-diamidino-2-phenylindole staining (DAPI; Invitrogen, Carlsbad, CA). FCM analyses were performed with Flowjo software (Tree Star, Ashland, OR).

Cytotoxic Assay

The cytotoxicity assay was performed by FCM as previously described.[16] Briefly, target cells labeled with 0.1 μmol/L carboxyfluorescein diacetate succinimidyl ester Cell Tracer Kit (Invitrogen) for 5 minutes at 37°C in 5% CO_2 were washed twice in phosphate-buffered saline solution, resuspended in complete medium, and counted with the use of trypan blue staining. The effector and target cells were coincubated at various ratios for 1 hour at 37°C in 5% CO_2. As a control, target cells or effector cells were incubated alone in complete medium to measure spontaneous cell death after DAPI was added to each tube. The data were analyzed with the use of Flowjo software. Cytotoxic activity was calculated as a percentage with the following formula: % cytotoxicity = [(% experimental $DAPI^+$ dead targets) − (% spontaneous $DAPI^+$ dead targets)]/[(100 − (% spontaneous $DAPI^+$ dead targets)] × 100.

ELISA

IFN-γ production of LMNCs during the culture was measured by enzyme-linked immunosorbent assay (ELISA) (Biolegend). Supernates collected after the incubation were stored at −80°C until further use. IFN-γ ELISA was performed according to the manufacturer's instructions.

Coculture with HCV Replicon Cells

The Huh7/Rep-Feo cell line (HCV replicon cells) was kindly provided by Dr N Sakamoto (Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo, Japan). The HCV subgenomic replicon plasmid, pRep-Feo, was derived from pRep-Neo (originally pHCVIbneo-delS).[33] pRep-Feo carries a fusion gene comprising firefly luciferase and neomycin phosphotransferase, as described elsewhere.[34,35] After culture in the pres-

ence of G418 (Invitrogen), Huh7/Rep-Feo cell lines showed stable expression of the replicons. We used transwell tissue culture plates (pore size 1 μm; Costar, Cambridge, MA) for coculture experiments. HCV replicon cells (10^5 cells) were incubated in the lower compartment with various numbers of lymphocytes in the upper compartment. The HCV replicon cells in the lower compartments were collected at 48 hours after the coculture for luciferase assays in duplicate with the use of a luminometer (TriStar LB 941; Berthold Technologies, Oak Ridge, TN) with the Bright-Glo Luciferase Assay System (Promega, Madison, WI).

Statistical Analysis

Data are presented as mean ± SEM. The statistical difference between results were analyzed by Student *t* test (2 tailed), using the Statistical Package for the Social Sciences (SPSS) software version 19 for Windows (IBM Corp, Armonk, NY). *P* values of ≤.05 were considered to be statistically significant.

RESULTS

Effect on the Surface Phenotype of LMNCs

In 5 LMNC preparations, the addition of OKT3 GMP CD3 to IL-2–stimulated LMNCs decreased $CD3^+CD56^-$ T cells to 0.2% ± 0.1% and 0.2% ± 0.1%, respectively, from the IL-2–only control value of 28.1% ± 12.3%. In contrast, $CD3^+CD56^-$ T cells were retained among LMNCs with the addition of antithymocyte globulin or alemtuzumab: 3.3% ± 2.0% and 17.2% ± 7.3%, respectively. The proportion of $CD3^-CD56^+$ NK cells increased by ~10% in all groups (Fig 1A).

Addition of OKT3 or GMP CD3 to IL-2–stimulated LMNCs maintained both activation and inhibitory markers on NK cells. Interestingly, the expressions of TRAIL, CD25 (IL-2αR), and CD132 (IL-2γR) were increased in the antithymocyte globulin group. Furthermore, both antithymocyte globulin and alemtuzumab completely blocked the expression of CD16 on NK cells (Fig 1B).

Cytotoxic Capacity

Cytotoxicity assays were performed with the use of freshly isolated cultured LMNCs as effectors and K562 cells as targets. Fig 2 shows freshly isolated LMNCs barely mediated cell death, whereas IL-2–stimulated LMNCs produced significant cytotoxicity. Although the ratios of $CD3^-CD56^+$ to $CD3^+CD56^+$ cells varied after treatment with various

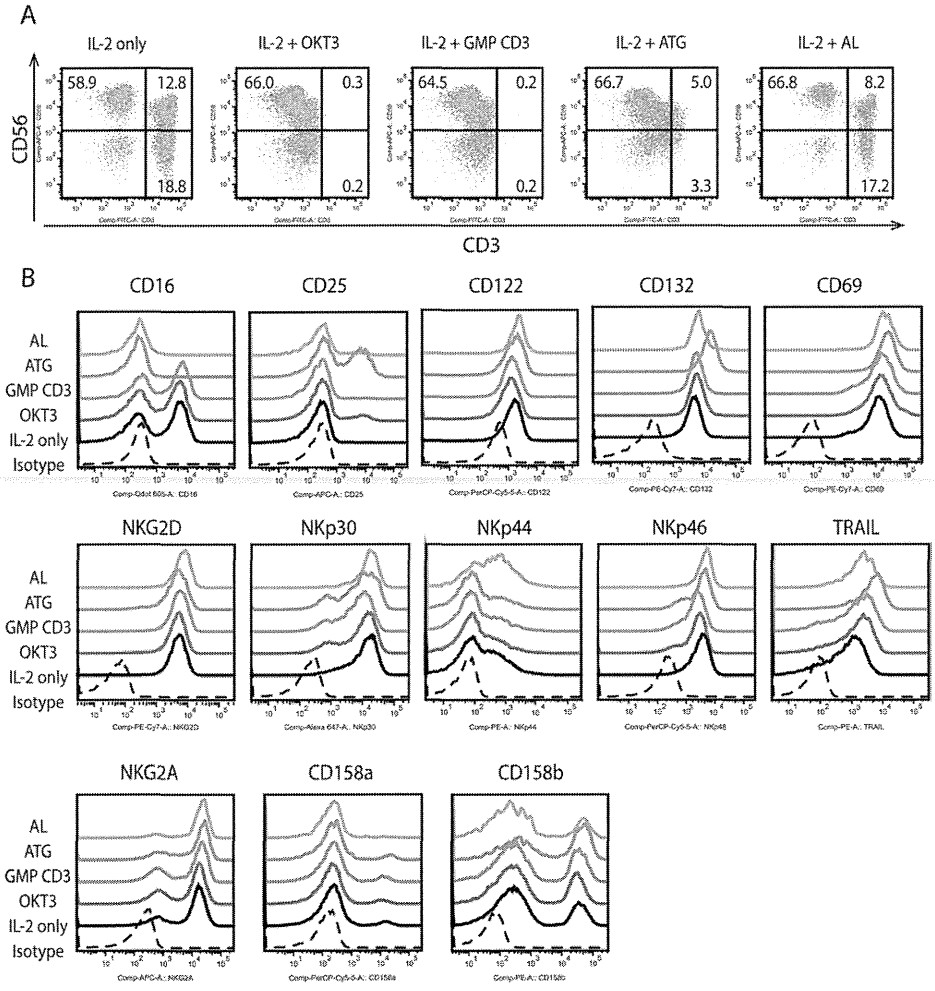

Fig 1. Effect of the T-cell depletion antibodies on the phenotypic characteristics of liver mononuclear cells (LMNCs). LMNCs obtained from cadaveric donors were stimulated with IL-2 (1000 U/mL) for 4 days. Anti-CD3 mAb (OKT3; 1 μg/mL), MACS GMP CD3 pure (GMP CD3; 1 μg/mL), antithymocyte globulin (ATG; 100 μg/mL), or alemtuzumab (AL; 100 μg/mL) was added to the culture medium 1 day before cell harvesting. (A) The LMNCs were stained with monoclonal antibodies against CD3 and CD56. The numbers indicate the mean percentages of the population. (B) Histograms show the logarithmic fluorescence intensities obtained on staining for each surface marker after gating on the $CD3^-CD56^+$ NK cells. Dotted lines indicate negative control samples with isotype-matched mAbs. The flow cytometry dot plot and histogram profiles represent 5 independent experiments. TRAIL, tumor necrosis factor–related apoptosis-inducing ligand.